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TRENDS IN MOLECULAR MEDICINE: REVIEW ARTICLE

Serotonin gene polymorphisms and bipolar I disorder: Focus on the serotonin transporter

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Abstract

The pathogenesis of bipolar disorder may involve, at least in part, aberrations in serotonergic neurotransmission. Hence, serotonergic genes are attractive targets for association studies of bipolar disorder. We have reviewed the literature in this field. It is difficult to synthesize results as only one polymorphism per gene was typically investigated in relatively small samples. Nevertheless, suggestive associations are available for the 5HT2A receptor and the serotonin transporter genes. With the availability of extensive polymorphism data and high throughput genotyping techniques, comprehensive evaluation of these genes using adequately powered samples is warranted. We also report on our investigations of the serotonin transporter, *SLC6A4* (17q11.1-q12). An insertion/deletion polymorphism (5HTTLPR) in the promoter region of this gene has been investigated intensively. However, the results have been inconsistent. We reasoned that other

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polymorphism/s may contribute to the associations and the inconsistencies may be due to variations in linkage disequilibrium (LD) patterns between samples. Therefore, we conducted LD analyses, as well as association and linkage using 12 polymorphisms, including 5HTTLPR. We evaluated two samples. The first sample consisted of 135 US Caucasian nuclear families having a proband with bipolar I disorder (BDI, DSM IV criteria) and available parents. For case-control analyses, the patients from these families were compared with cord blood samples from local Caucasian live births (n=182). Our second, independent sample was recruited through the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD, 545 cases, 548 controls). No significant associations were detected at the individual polymorphism or haplotype level using the case-control or family-based analyses. Our analyses do not support association between *SLC6A4* and BDI families. Further studies using sub-groups of BDI are worthwhile.

Key words: *Association, bipolar disorder, genetic, polymorphism, serotonin, serotonin transporter*

Introduction

Twin, adoption and family studies have suggested substantial genetic contributions to the etiology of bipolar I disorder (BDI) (1). It has proven difficult to identify individual genetic risk factors, possibly because the contribution of such factors is relatively small, and complex interactions may be involved (2). Complementing conventional linkage analyses, association studies have become increasingly popular for gene mapping studies of BDI as they may detect susceptibility loci with small effects (3).

There is suggestive evidence implicating dysregulation of serotonin (5-hydroxytryptamine, 5-HT) in the pathogenesis of a range of psychiatric disorders, including mood disorders (4–6). Serotonergic neurons are also targets for antidepressants (7–9). Hence genes regulating the synthesis, transport, and metabolism of 5-HT, as well as genes encoding multiple 5-HT receptors have been extensively analyzed. In the following section, we review peer-reviewed publications in English describing genetic association studies of serotonergic polymorphisms and BDI. Our association and linkage analyses of the serotonin transporter (*SLC6A4*) follow. Serotonin related genes are also of interest for pharmacogenetic analyses, but the majority of these studies have focused on depression and therefore are not reviewed here (10–12).

Tryptophan hydoxylase *(TPH)*

TPH encodes the rate-limiting enzyme that catalyzes the oxidation of tryptophan to 5-hydroxytryptophan and is thus critical for the synthesis of 5-HT. The gene for human TPH has been cloned (13) and mapped to chromosome 11p14-p15.3 (14). Recently, a second isoform of TPH has been discovered and has been named TPH2 (15). *TPH2* is localized to chromosome 12q21.1. TPH2 is preferentially expressed in the brain, whileTPH1 in mainly expressed in peripheral tissues (15,16).

Published association studies for the *TPH1* gene and bipolar disorder have been inconsistent. A significant association with an intronic A218C polymorphism was first reported in a French sample. In this case-control study, 152 patients (103 patients with BDI and 49 patients with BDII) were compared with 94 healthy control individuals (odds ratio, OR = 3.96 for probands homozygous for allele 218A) (17). However, significant associations were not detected in subsequent studies using case-control or family-based samples (18–22). Most of the studies lacked power to replicate the initial association. Recently, an association with a functional polymorphism of TPH2 and major depressive disorder was reported (23). A subsequent study did not detect association with bipolar disorder or suicidal behavior (24). Hence, it is difficult to make a definitive pronouncement about this association. This is a recurring theme for the other candidate genes reviewed below.

Serotonin 2A receptor *(HTR2A)*

HTR2A is thought to play a key role in the action of antidepressants such as selective serotonin reuptake inhibitors (SSRIs) (4). Several polymorphisms of *HTR2A*, localized to chromosome 13q14-q21 have been extensively analyzed in genetic association studies of BDI. A number of significant associations have been published. In one of the earliest studies, the frequency of allele C102 at the 102T/C (rs6313) single nucleotide polymorphism (SNP) was elevated among Caucasian cases compared with unrelated controls (OR 1.79) (18). The 102T/C SNP is localized to exon 1 and represents a synonymous substitution. Another association has been observed at -1438A/G (rs6311, a SNP localized to the putative promoter region) in separate samples of Caucasian and Korean ethnicity, albeit with different alleles (25,26). However, the majority of studies did not detect associations at *HTR2A* (27–33) (see 34).

Most studies investigated cases and unrelated controls, so the significant associations could be attributed to population sub-structure secondary to population admixture (35). In other words, differing

ethnic backgrounds, rather than the disorder of interest may account for the case-control difference. On the other hand, the majority of the studies with non-significant associations investigated only one polymorphism and did not investigate all the documented variants. Thus, it is difficult to conclude that the negative reports represent absence of association. Indeed, when we investigated nine SNPs, a significant association at 1354C/T was detected using case-control as well as family-based analyses (34). In addition, an association with 516C/T was detected using case-control, but not family-based analyses. Though 1354C/T represents a synonymous substitution, it is in significant LD with 102T/C. Comprehensive analyses of all polymorphisms at this locus are warranted, using samples with adequate power.

Key messages

- Dysfunction of the serotonergic neurotransmission has long been suggested to play a role in the pathogenesis of bipolar disorders.
- Extensive research was conducted analyzing polymorphisms of several serotonin genes. However, consistent associations did not emerge. The inconsistencies may be due to inadequately powered samples and/or insufficient analysis of polymorphisms at these loci.
- Associations between bipolar I disorder and polymorphisms of the serotonin transporter gene were not detected.

Serotonin 2C receptor *(HTR2C)*

HTR2C has been mapped to chromosome Xq24 (36) and is a plausible candidate as it is involved in affective/perceptual states and endocrine function (37). An exonic polymorphism (Cys23Ser) was investigated among 88 bipolar patients and 113 controls from Spain, but significant case-control differences were not detected. However, a suggestive association with the Ser23 allele was observed among female cases ($P = 0.04$, not corrected for multiple comparisons) (38). These results were supported by a later study of 42 bipolar patients and 40 healthy controls from Croatia ($P = 0.051$) (39). However, association could not be detected in two studies analyzing two polymorphic di-nucleotide repeats in the promoter region, namely (GT)12-18 and (CT)4-5 (40,41). Based on these results, the Cys23Ser SNP was analyzed in a large multi-center study comprising 649 bipolar patients and 901 normal controls from nine European countries. A significant excess of Ser23 allele carriers in patients compared to controls was observed (chi square = 5.45, df 1, P=0.02) (42). However a significant variation in the allele frequency for Ser23 was noted across population groups included in the study, raising the possibility of a 'spurious' association attributable to ethnic admixture. Associations at *HTR2C* have not been detected in other, smaller samples (18,43). Thus, the putative associations are reminiscent of the *HTR2A* association studies and merit comprehensive evaluation.

Other serotonergic receptors

The HTR1A, HTR1D alpha, HTR1D beta, HTR3A, HTR4 and HTR7 receptors have all been investigated in one or two studies (44,18,45). For example, in a case-control study from Japan (53 patients, 187 controls), significant association of four polymorphisms at *HTR4* gene with bipolar disorder was reported (OR 1.5–2) (45). These associations were supported by transmission disequilibrium test (TDT) analysis in 69 multiplex pedigrees from the National Institute of Mental Health (NIMH) Genetics Initiatives Bipolar Pedigrees. In view of the suggestive associations, further analyses at *HTR4* are warranted.

Monoamine oxidase (*MAO)*

Monoamine oxidase type A (*MAOA*) and type B (*MAOB*) are enzymes that catalyze the degradation of biogenic amines including serotonin (46). Hence, they are of great interest in psychiatric research and may even mediate the pathogenesis of affective disorders (47). Genes encoding these enzymes are tightly linked and arranged in a 'tail-to-tail' pattern on chromosome Xp11.23-11.4 (48–50).

Several polymorphisms of *MAOA* have been investigated in genetic association in bipolar disorders. They include a di-nucleotide repeat polymorphism in the second intron (MAOA-CA) (51), an *Fnu*4HI restriction fragment length polymorphism (RFLP) in exon 8, an RFLP resulting from single base substitution in the third base of a triplet codon (G → T at position 941, also called 'MAOA-RFLP) (52) and variable number of tandem repeats (VNTR) polymorphism in intron 1 ('MAOA-VNTR') (53). A significant association was detected at three polymorphisms in a sample of 57 British bipolar patients and 59 unaffected controls. A significant association with alleles of

a MAOB polymorphism was not detected (dinucleotide sequence in the 2nd intron; MAOB-GT) (54). These promising results were supported by three other studies. The first reported significant associations with the MAOA-CA marker in a sample of 58 Japanese bipolar patients and 68 controls ($P = 0.029$) (55). An association with the same polymorphism was also detected in another sample of UK patients, though an association with the Fnu4HI RFLP was not detected (56). In France and Switzerland, 272 patients with bipolar disorder and 122 healthy subjects were investigated for the MAOA-CA repeat, the MAOA-RFLP, and the VNTR. A significant difference in the distribution of the alleles for the MAOA-CA repeat was observed between the female bipolar patients and the comparison group (57). In contrast, no evidence for association was found with MAOA-CA, *Fnu*4H1 RFLP or MAOA-VNTR in another British sample composed of 84 bipolar patients (70 of them had BDI) and 84 controls (58). The same *MAOA* polymorphisms, as well as an additional *MAOB* marker were investigated in a Japanese sample consisting of 60 patients and 100 controls. Significant associations were not detected (59). Another case-control study from Japan (132 patients, 169 controls) did not detect associations with two polymorphisms *(T/941/G* and *A/1609/G)* (60). No association could be found after analysis of the MAOA-LPR polymorphism in a larger sample from Germany (174 patients with affective disorder, 229 controls) (61). A number of other investigators have analyzed MAO polymorphisms using family-based controls and these have been published, although none of their findings were significant (62–64) (21). Thus, there are no credible associations at *MAOA* or *MAOB*, though it should be noted that many of the replication samples were underpowered and polymorphisms at these genes have not been investigated comprehensively. A similar conclusion was reached in a recent review (65).

Serotonin transporter (*5-HTT*, *hSERT*, *SLC6A4)*

The serotonin transporter has received considerable attention, as it mediates serotonin reuptake into presynaptic neurons. It is also the likely site of action of tricyclic antidepressants and serotonin re-uptake inhibitors. Hence, the gene for the serotonin transporter (*SLC6A4*), which maps to chromosome 17q11.1-q12, is a plausible candidate in the pathogenesis of BDI.

SLC6A4 polymorphisms have been investigated widely among cases and population-based controls (Table I). A study in the UK involving Caucasian BDI patients and unrelated controls first reported an association with allele 12 of a VNTR in the second intron (66). These results received support from another study conducted independently in the UK (67). This group also reported association with bipolar affective puerperal psychosis (68). A study from France did not detect the association with bipolar disorder, but reported association with increased homozygosity for the short variant of a 44 bp insertion/deletion polymorphism in the 5' untranslated region (5' UTR) denoted as 5-HTTLPR (69). This polymorphism is considered to be important as it affects the transcription, and hence possibly the function of the serotonin transporter (70). The results from the French study are also plausible because the VNTR and 5-HTTLPR polymorphisms are in strong linkage disequilibrium (LD) (71). In other words, allelic status at the VNTR and 5-HTTLPR are significantly correlated at the population level. A third UK study examined both polymorphisms. Though significant associations were not detected in this sample, meta-analysis of published studies suggested a significant association with allele 2 (the shorter allele) at the (5-HTTLPR) marker (72) (Table I). A recent meta-analysis study applied to case-control studies of 5-HTTLPR revealed that the short allele(s) of the 44-bp ins/deletion polymorphism showed a modest but significant association for bipolar disorder (OR = 1.13, $P = 0.001$) (73). Another recent Japanese study not included in the meta-analysis, however, did not reveal a significant association (74).

A number of family-based association studies involving *SLC6A4* polymorphisms have also been reported. A study involving 122 case-parent trio families did not report significant association with either polymorphism using the transmission disequilibrium test (TDT). Since this sample included cases previously reported (66), analysis was repeated among 98 independently ascertained families. Significant excess transmission of the 12-repeat VNTR allele was observed in this sub-group. The TDT results for the VNTR polymorphism were supported by haplotype-based analyses involving the VNTR and 5-HTTLPR polymorphisms (71). Another study reported a significant TDT result for the 5HTTLPR polymorphism in BDI (75). Significant transmission distortion suggests association in the presence of linkage, providing further credence to the results. Non-significant TDT results have also been reported at *SLC6A4* (76,77,21).

Table I. Published BD1 case-control association studies using *SLC6A4* polymorphisms.

Reference	Marker	Ethnicity	Cases	Controls	Dx	Comment
(66)	VNTR	Caucasian+not specified	252	187	DSM-IV	Association with 12 repeat allele ($P = 0.00048$, allele-wise)
(67)	VNTR	Caucasian	124	121	DSMIII-R	Association with 12 repeat allele
(104)	VNTR	Japanese	98	137	RDC	NS
(105)	VNTR	Caucasian	79	86	DSMIII-R	NS
(99)	VNTR 5-HTTLPR	Caucasian	79	294	DSM-IV SADS-LA	NS
(101)	VNTR 5-HTTLPR	Spanish	88	113	DSMIII-R	NS
(72)	VNTR 5-HTTLPR	Sample 1 Caucasian Sample 2 Caucasian	87 375 392	174 772 739	RDC DSM-IV	NS Meta-analysis: Significant association with 5-HTTLPR allele 2 (chi-square = 3.68, d.f. = 1, $P = 0.049$, OR 1.21)
(106)	VNTR 5-HTTLPR	British Caucasian	171	121	DSM-IV	NS with 5-HTTLPR. VNTR, Association with 12 repeat allele ($P = 0.031$)
(69)	5-HTTLPR VNTR	Caucasian	208	99	DSM-IV	Association with homozygosity for short variant of 5-HTTLPR (chi-square = 4.68, d.f. = 1, $P = 0.03$, OR 2.11)
(107)	5-HTTLPR	Mixed*	47	98	DSM-IV	NS
(18)	VNTR 5-HTTLPR	Sample 1 Mixed** Sample 2 Caucasian	103 109	103 109	RDC	NS
(97)	5-HTTLPR	Colombian	103	112	DIGS	NS. Excess short allele in younger cases and in cases with psychotic symptoms.
(108)	VNTR	Indian	50	50	ICD-10	NS
(98)	5-HTTLPR	Caucasian	572	821	SCAN SADS-LA	NS
(74)	5-HTTLPR VNTR+10 SNPs	Japanese	61 (BD1)	288	DSM-IV	NS

Dx = diagnostic criteria; DSM = Diagnostic and Statistical Manual for Mental Disorders; RDC = research diagnostic criteria; DIGS = Diagnostic Interview for Genetic Studies; SCAN = Schedule for Clinical Assessment of Neuropsychiatry; SAD-LA = Schedule for Affective Disorders and Schizophrenia–lifetime version; VNTR = variable number of tandem repeat polymorphism in the second intron; 5HTTLPR = insertion/deletion polymorphism in the 5' upstream regulatory region; NS = ot significant; BD1 = bipolar 1 disorder; * 80% Caucasian and 20% African/Brazilian; ** 95% Caucasian, 3% Oriental, and 2% Native Canadian.

Thus, a number of reports suggest associations with a functional polymorphism. However, the non-significant results need to be considered also. There are several explanations for the inconsistent associations. First, the sample sizes of the published studies are relatively small, so each sample had limited power to detect small effects on liability. Second, most of the studies have examined one or two polymorphisms, which limits the power to detect association if the measured polymorphisms are only in LD with polymorphisms having a direct effect on liability. Indeed, it is now known that 5-HTTLPR is not a binary insertion/deletion polymorphism, but involves a more complex repeat structure (78). Since the majority of studies employed unrelated controls, varying levels of population sub-structure could also explain the inconsistencies (79).

To evaluate these possibilities, we investigated twelve polymorphisms at *SLC6A4*. To increase the informativeness of the available polymorphisms, we conducted additional haplotype-based analyses. We employed case-control, as well as family-based analyses in order to understand artifacts due to ethnic substructure.

Materials and methods: Clinical

Pittsburgh sample

Probands with bipolar I disorder (DSM IV Criteria), and available parents were included. Details of ascertainment and diagnosis are described elsewhere (34) (80). Briefly, consenting patients were interviewed using the Diagnostic Interview for Genetic Studies (DIGS), a structured diagnostic interview schedule (81). Additional clinical information was obtained from available clinical records and from relatives. This information was synthesized and a consensus diagnosis assigned. The parents provided blood samples, but detailed diagnostic interviews were not conducted.

Unscreened controls. Cord blood samples were obtained from live births at Magee-Women's Hospital, Pittsburgh and served as unscreened,

population-based controls. No information apart from ethnicity was available for these samples.

STEP-BD sample

Patients. We obtained genomic DNA samples from patients with a baseline diagnosis of bipolar I disorder (DSM IV criteria) recruited through the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD). This is a longitudinal study aimed at improving treatment for Bipolar Disorder (82). STEP-BD used a network of eighteen U.S. treatment centers for standardized evaluation and treatment of patients including interview schedules based on the Structured Clinical Interview for DSM-IV (SCID), as well as the Mini-International Neuropsychiatric Interview (MINI) (83,84).

Unscreened controls. Control samples were not collected as part of the STEP-BD cohort. For comparison with the STEP-BD cases, additional cord blood samples were obtained from Pittsburgh, independent of the unscreened 'Pittsburgh' samples described above.

All participants reported Caucasian ethnicity (maternal report for neonatal samples). We obtained written informed consent from participants, according to the guidelines of the University of Pittsburgh Institutional Review Board (IRB), as well as IRBs at relevant STEP-BD recruitment sites.

Materials and methods: Laboratory

Genomic DNA was extracted from venous blood samples using the phenol chloroform method.

SNP selection

Fifteen polymorphisms were selected over a 38 kb genomic region spanning *SLC6A4*. These polymorphisms were identified by other investigators following bi-directional sequencing of 10 unrelated autism probands (85). Among the polymorphisms that were analyzed initially; three were discarded due to inconsistent genotyping assays (rs6352 A/C, rs2020941 T/C and rs2020936 T/C). Thus, 12 polymorphisms were used in the analyses. They included the insertion/deletion polymorphism (5-HTTLPR), as well as 11 additional SNPs, which are listed in public databases (http://www.ncbi.nlm.nih.gov/genome/guide/human/). Genomic DNA from a panel of eight individuals was re-sequenced for each of the 11 SNPs. For 5HTTLPR, we re-sequenced 48 individuals. These samples served as positive controls for subsequent genotype assays.

Genotype assays

Variation in 5HTTLPR was analyzed by re-sequencing, as well as a polymerase chain reaction (PCR) based assay (86). Genotype assays for the SNPs were based on multiplex PCR followed by single base extension analysis (SnaPShot assays, ABI Inc; details available online at www.pitt.edu/~nimga, supplemental Table I).

All allele calls were re-checked independently, blind to diagnostic status. Genotypes were then evaluated for Mendelian consistency using PedCheck in the family-based sample (87). In case of any discrepancy, samples were re-typed. All clinical and genetic data were double-checked to guard against data entry errors.

Statistical analysis

We evaluated linkage disequilibrium (LD), defined as the non-random association of variants at linked loci (88). Several measures of LD are available, the classic measure being 'D' (89). As D is influenced by allele frequencies, we used two scaled measures, namely D' and r^2 (90–92). The latter was estimated using a method based on a hierarchical clustering procedure called 'H-clust'. This method also enables selection of 'tag SNPs', i.e. SNPs that can be used to successfully predict unmeasured SNPs over a selected region and thus can be said to represent polymorphism in that region (93). The software program GENEHUNTER (GH) was used for TDT analysis (94). For family-based association analyses, we used the 'uncertain haplotypes' option in the TDTPHASE program to analyze SNP based and haplotype based data (software package UNPHASED) (95). For case-control comparisons, we utilized the Armitage trends test to evaluate genotype frequency differences between our cases and unrelated controls (96).

Results

The Pittsburgh family-based sample included 135 cases; both parents were available for 61 families, while a single parent was available for the remaining 74 families. All the patients from these families were compared with 182 controls. The STEP-BD sample included 545 BDI cases and 548 controls. No Mendelian inconsistencies were noted for the

family based samples. All genotype distributions were in Hardy-Weinberg equilibrium (HWE) for cases, parents and neonatal controls, with the exception of 5-HTTLPR. A heterozygote deficit was noted for this polymorphism among the STEP-BD cases ($P = 0.005$). Since departure from HWE can arise due to genotyping or book keeping errors, all genotypes from the STEP-BD samples were re-read. In case of ambiguity, samples were genotyped again. Results from the final assays are presented below.

Genomic structure of 5-HTTLPR:

Consistent with published results, we detected a complex polymorphism after re-sequencing 48 individual samples (86). The polymorphism consists of a 44 bp repeat in the promoter region. Further, we identified another SNP flanking 5HTTLPR, localized to the repeat motif (rs25532). In our sample, rs25532 is homozygous among all individuals homozygous for the 5HTTLPR insertion.

LD structure:

The relative positions of the other polymorphisms are displayed in Table II. Pair-wise LD patterns were evaluated between the polymorphisms among Pittsburgh patients, their parents, the controls, and the STEP-BD controls, and tag SNPs selected by H-clust with $r^2 = 80\%$ (93) (Figures 1 and 2; Table IV; haploview analysis available online at www.pitt.edu/~nimga supplemental Figures 1 and 2)

Using Haploview, two distinct 'blocks' were identified; the first composed of six SNPs, (rs140701, rs2020942, rs6354, rs2020939, rs2020937 and rs2066713) and the second composed of four other SNPs (rs2020935, rs2020934, rs2020933 and rs2020930). The remaining two SNPs, including 5HTTLPR, did not form LD blocks, although 5HTTLPR is in significant LD with rs2020934 (91).

Analyses using 'H-clust' were similar overall to the 'blocks' outlined using Haploview, although there were some distinctions. Close clustering was observed for five of the six SNPs that composed 'block 1', but the remaining SNP (rs6354) did not cluster with the other SNPs, unlike the Haploview analysis (see Figure 2 in this text, and Figure 1 online www.pitt.edu/~nimga). There was more disparity between these two methods with regard to 'block 2'. Unlike the D' analyses, rs2020934 was not closely related to rs2020935 or rs2020933. Instead, it clustered with 5HTTLPR.

Association and linkage analysis:

Pittsburgh sample. No significant associations were detected after comparing genotype and allele distributions for each polymorphism between patients with BDI and neonatal controls. Consistent with these results, no significant transmission distortion was observed using the TDT for 5HTTLPR, individual SNPs or 'sliding window' haplotypes based on 2, 3 or 4 contiguous polymorphisms (Table II). Significant associations were also not detected using the UNPHASED program at the SNP or at the haplotype level. An excess of the short allele of 5HTTLPR was previously observed in cases

Table II. Association and linkage analysis of ploymorphisms at *SLC6A4* using Pittsburgh BD1 sample.

	Marker	Allele	Position in bp*	Frequency of less common allele		Trends test (*P* value)	TDT *T/NT*
				cases	controls		
1	rs140701	C/T	3275525	0.406	0.425	0.668	28/28
2	rs2020942	G/A	3283907	0.399	0.377	0.586	25/26
3	rs6354	G/T	3286891	0.192	0.198	0.839	16/17
4	rs2020939	G/A	3287725	0.414	0.419	0.910	29/28
5	rs2020937	A/T	3287750	0.396	0.377	0.640	26/25
6	rs2066713	G/A	3288658	0.399	0.375	0.539	25/26
7	rs2020935	A/T	3298448	0.077	0.058	0.340	5/5
8	rs2020934	C/T	3298453	0.496	0.477	0541	16/26
9	rs2020933	A/T	3298748	0.06	0.061	0.954	4/4
10	rs25532	C/T	3301163	0.162	0.141	0.483	11/11
11	5-HTTLPR	L/S	3301301...3301344	0.452	0.458	0.884	18/24
12	rs2020930	G/A	3303034	0.042	0.036	0.658	4/3

*Genomic sequence, including exons and introns (www.ncbi.nlm.nih.gov, build 34, version 2). TDT = transmission disequilibrium test; T/NT = transmitted/non-transmitted, this refers to the first allele in column 3; L/S = long allele/short allele; No significant differences were observed using Trends test for case-control analysis or TDT for family-based analysis.

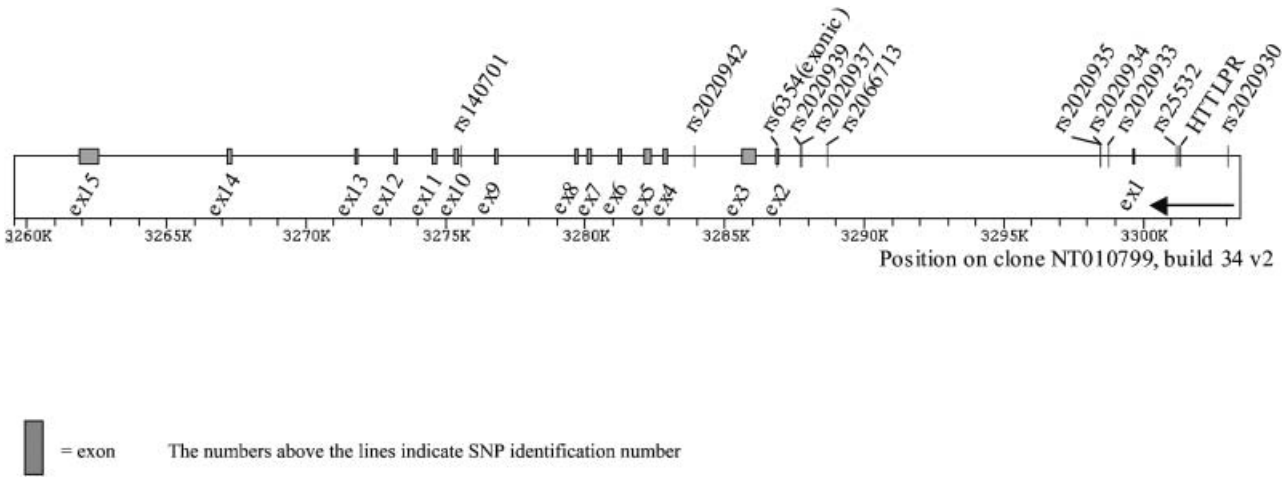


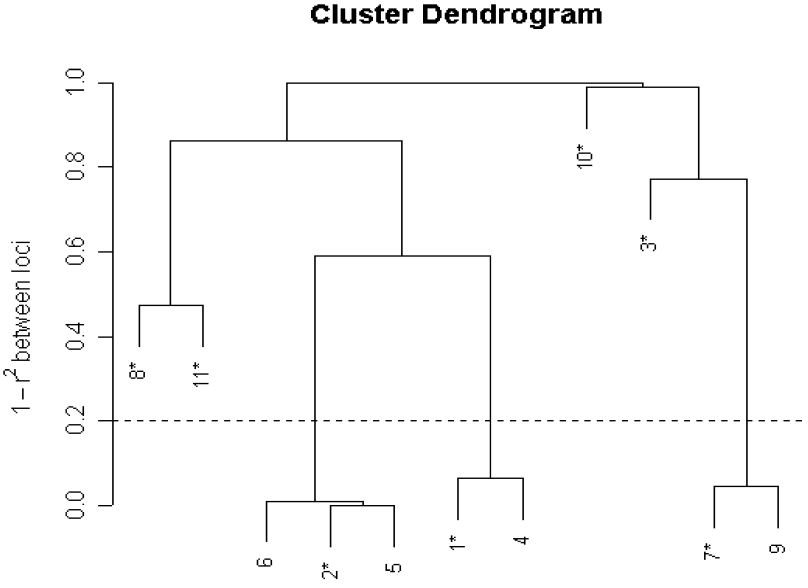
Figure 1. Structure of *SLC6A4* gene and polymorphisms analyzed.

with psychotic symptoms (97). However, additional analyses comparing our Pittsburgh BDI cases with psychotic features against neonatal controls did not detect this effect (data not shown).

STEP-BD sample. Allele frequencies for individual polymorphisms were consistent with those observed in the Pittsburgh samples. The case-control comparisons conducted in the Pittsburgh sample were repeated in the STEP-BD sample. No significant associations were detected using the larger STEP-BD sample at the individual polymorphism or at the haplotype levels (Table III).

Discussion

Our review of published studies did not reveal a clear pattern of association with any serotonergic (polymorphism, though a number of intriguing and plausible associations have been reported. Most of these studies were published prior to initiation of the Hapmap project (http://www.hapmap.org/). Hence only one or two polymorphisms were typically analyzed at each gene. As a large number of polymorphisms are now documented for most of these genes, it may be appropriate to conduct analyses using such polymorphisms. It will be economical to identify 'tag SNPs' that can represent



* Tag SNPs selected by H-Clust analysis. Legend for *SLC6A4* markers: 1 = rs14070; 2 = rs2020942; 3 = rs6354; 4 = rs202093; 5 = rs2020937; 6 = rs2066713; 7 = rs2020935; 8 = rs2020934; 9 = rs2020933; 10 = rs25532; 11 = 5HTTLPR; 12 = rs2020930.

Figure 2. LD Pattern using H-Clust analysis: Controls from the Pittsburgh sample.

Table III. Association analysis of polymorphisms at *SLC6A4* using the STEP-BD sample.

	Marker	Allele	Position in bp*	Frequency of less common allele		Trends test (*P* value)
				cases	Controls	
1	rs140701	C/T	3275525	0.432	0.427	0.816
2	rs2020942	G/A	3283907	0.391	0.384	0.736
3	rs6354	G/T	3286891	0.183	0.202	0.288
4	rs2020939	G/A	3287725	0.432	0.427	0.812
5	rs2020937	A/T	3287750	0.394	0.383	0.606
6	rs2066713	G/A	3288658	0.392	0.383	0.640
7	rs2020935	A/T	3298448	0.057	0.067	0.307
8	rs2020934	C/T	3298453	0.488	0.480	0.705
9	rs2020933	A/T	3298748	0.049	0.066	0.093
10	rs25532	C/T	3301163	NA	NA	NA
11	5-HTTLPR	L/S	3301301...3301344	0.439	0.428	0.629
12	rs2020930	G/A	3303034	0.033	0.042	0.278

*Genomic sequence, including exons and introns (www.ncbi.nlm.nih.gov, build 34, version 2). L/S = long allele/short allele; NA = not analyzed. No significant differences were observed using Trends test for case-control analysis.

a significant proportion of the variations across the entire gene, as was demonstrated here for *SLC6A4* (93). Here, we have suggested a method for selection of tag SNPs. In addition, much larger samples will soon become available to the scientific community (82) (http://www.nimh.nih.gov/researchfunding/geneticsinitiative.cfm).

In view of these concerns, we conducted a more comprehensive evaluation of the serotonin transporter (*SLC6A4*). In addition to the well-known 5-HTTLPR marker, we included 11 additional SNPs spaced across the gene. Our initial analyses revealed a lack of significant LD between 5-HTTLPR and most of the other SNPs analyzed here, emphasizing the need for evaluation of multiple polymorphisms. To understand variations due to differences in control selection, we conducted association analyses using case-control, as well as family-based designs. We did not detect a significant association with any of the *SLC6A4* polymorphisms in two independent samples composed of cases and community based controls. Our case-control results are consistent with other previous negative studies (98–101,18). In addition, we did not find significant associations using the TDT, in agreement with two prior reports (76,77).

Some limitations of our analyses should be noted. Our analyses were restricted to individuals reporting Caucasian ancestry. Our 'Pittsburgh sample' was relatively small, particularly for the family-based analyses. The requirement for heterozygous parents restricts the number of family units that can be used for TDT analyses. The TDT also has limited usefulness and has reduced power when only one parent is available in a family unit (102,103). Since a proportion of our sample included single-parent family units, we also compared our cases with unrelated controls.

Departure from Hardy Weinberg equilibrium was noted only among the STEP-BD patients with regard to 5-HTTLPR genotypes. We note that the *P* value (0.005) does not remain significant following correction for multiple comparisons (12 polymorphisms; two sets of patients, two set of controls and one set of parents). Nevertheless, concerns about genotype errors lead us to re-evaluate our results. As such errors do not explain the departure from HWE, we attribute it to chance variation.

Since we did not re-sequence *SLC6A4* comprehensively in our samples, it is possible that other unidentified polymorphisms may yet be associated with BDI at *SLC6A4*. Some investigators have reported an association with a VNTR in the second intron. This polymorphism was not analyzed here as it is reported to be in strong LD with 5HTTLPR (71). At present, twelve SNPs have been analyzed by the HapMap project. Haploview analysis revealed that the SNPs form two blocks (data not shown). Four SNPs present in these blocks were also analyzed in our set. Thus, our set appears to have a reasonable representation of polymorphisms at *SLC6A4*, at least with respect to 'common' SNPs. In addition, our set also includes 'tag' SNPs from H-Clust analysis (Figure 2). The disparity between the cluster analyses and the Haploview analyses is due to quite different approaches and assumptions about the pattern of LD in the genome. For example, two point LD analysis, on which Haploview is based, is highly dependent on sample size, unlike H-Clust analysis. Analyses of LD suggest that the genome does not show perfect block like structure, but rather can show a very patchy pattern of LD (93).

Table IV. Linkage disequilibrium across *SLC6A4* using D' and r^2 values among Pittsburgh BDI cases.

Polymorphism	rs140701	rs2020942	rs6354	rs2020939	rs2020937	rs2066713	rs2020935	rs2020934	rs2020933	rs25532	5hTTLPR	rs2020930
rs140701	\	0.98	0.94	0.99	0.98	0.98	0.74	0.8	0.86	0.59	0.45	1
rs2020942	0.48	\	0.95	1	1	1	0.76	0.79	0.35	0.33	0.63	0.44
rs6354	0.13	0.13	\	0.95	0.95	0.95	0.73	0.11	0.73	1	0.05	0.79
rs2020939	0.97	0.5	0.13	\	1	1	0.74	0.78	0.86	0.57	0.45	1
rs2020937	0.49	0.99	0.14	0.51	\	1	0.76	0.79	0.37	0.34	0.63	0.46
rs2066713	0.49	0.99	0.14	0.51	1	\	0.76	0.79	0.37	0.34	0.63	0.46
rs2020935	0.03	0.03	0.2	0.03	0.03	0.03	\	1	0.94	1	0.74	1
rs2020934	0.49	0.4	0	0.48	0.41	0.41	0.06	\	1	0.95	0.76	1
rs2020933	0.03	0.01	0.16	0.03	0.01	0.01	0.8	0.05	\	1	0.68	1
rs25532	0.08	0.01	0.03	0.07	0.01	0.01	0.01	0.16	0.01	\	0.91	1
5hTTLPR	0.2	0.2	0	0.2	0.2	0.2	0.03	0.44	0.02	0.19	\	0.58
rs2020930	0.03	0.01	0.11	0.03	0.01	0.01	0.48	0.03	0.57	0.01	0.01	\

D' and r^2 values are shown above and below the diagonal (\) respectively (88).

Conclusion

There is suggestive evidence for dysfunction in serotonergic neurotransmission among patients with BDI. Hence, it is appropriate to investigate genetic associations with serotonergic genes. Polymorphisms of several such genes have been analyzed, but consistent associations have not emerged. The inconsistencies could be due to differences in sample size, number of markers analyzed, artifacts introduced by ethnic admixture, heterogeneity or may indicate the absence of a 'true' association. The time may be ripe for comprehensive analysis of these genes systematically and comprehensively, using adequately powered samples.

Our investigation of Caucasian cases, controls, and parents did not reveal significant association between serotonin transporter (SLC6A4) gene polymorphisms and BDI. Our results are concordant with a majority of prior reports. Though our analyses did not reveal associations with *SLC6A4*, associations at this locus among sub-groups of BDI are possible, such as those based on mixed or rapid cycling, clinical course, vulnerability to adverse life events, or response to relevant therapies.

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